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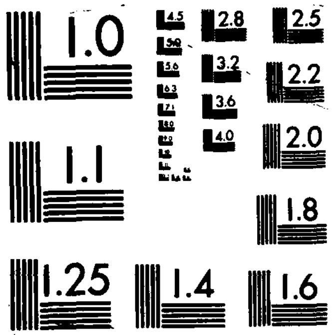

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RIFT VALLEY FEVER VIRUS: MOLECULAR BIOLOGIC STUDIES OF THE M SEGMENT RNA FOR APPLICATION IN DISEASE PREVENTION

ANNUAL REPORT

MARC S. COLLETT, Ph.D.

AUGUST, 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5226

Molecular Genetics, Inc.
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Various aspects of the genomic M segment RNA of RVFV were investigated. The messenger RNA (mRNA) of this segment was found to lack at its 3' end approximately 112 nucleotides found at the 5' end of the M genomic RNA. The 5' end of the mRNA possessed all of the sequences present at the 3' end of the M RNA, but was further extended beyond the end of the genome by about 12-14 nucleotides. Four distinct antigenic determinants along the G2 glycoprotein were localized to small peptide regions of between 11 and 34 amino acids. These epitopes were defined by four monoclonal antibodies (mAbs), three of which were capable of neutralizing virus infectivity; one was non-neutralizing. This and other information were used to design and construct plasmids that, when introduced into *E. coli*, directed the synthesis of novel polypeptides containing glycoprotein G2 sequences. These polypeptides are under evaluation for their ability to elicit protective immunity in lab animals against RVFV challenge. Live recombinant vaccinia viruses have been constructed that possess the entire coding regions for both RVFV glycoproteins. These viruses expressed and correctly processed the G2 and G1 proteins. Mice immunized with these viruses developed high titers of virus neutralizing antibodies and were protected from lethal RVFV challenge.

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SUMMARY

The purpose of this work is to elucidate the molecular and biological properties of the genomic M segment RNA of RVFV which encodes the viral envelope glycoproteins. Progress along several objectives is summarized in this report. Using S1 nuclease mapping and oligonucleotide primer extension procedures, it was found that the messenger RNA (mRNA) of the M segment lacks at its 3' end approximately 112 nucleotides present at the 5' end of the genomic RNA. The 5' end of the mRNA possessed all of the sequences present at the 3' end of the M RNA, but was further extended beyond the end of the genome by about 12-14 nucleotides. These results provide essential information necessary for understanding the expression strategy of this viral RNA segment. Using recombinant DNA techniques and *E. coli* plasmid expression cloning systems, four distinct antigenic determinants along the G2 glycoprotein were localized to small peptide regions of between 11 and 34 amino acids. These epitopes were defined by three monoclonal antibodies (mAbs) capable of neutralizing virus infectivity and one non-neutralizing mAb. This, along with other information, was used to design and construct plasmids that, when introduced into *E. coli*, directed the production of novel polypeptides containing G2 sequences. These polypeptides will be evaluated as subunit immunogens for their ability to elicit virus neutralizing antibodies and protective immunity in lab animals. An alternative approach to RVF vaccine development involves the use of live recombinant vaccinia viruses. Such recombinant viruses have been constructed using genetic engineering techniques that possess the entire coding regions for both RVFV glycoproteins. These viruses expressed and correctly processed the G2 and G1 proteins. Mice immunized with these viruses developed high titers of virus neutralizing antibodies and were protected upon lethal RVFV challenge. These RVFV-vaccinia recombinant viruses hold excellent promise for future vaccine development.

FOREWORD

In performing the recombinant DNA experiments described in this report, the investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

In conducting laboratory animal immunization experiments described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication N. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

A. Introduction/Background. Rift Valley fever is an important disease of domestic livestock populations in much of sub-Saharan Africa, causing acute febrile disease, abortions, and death in cattle and sheep. Rift Valley fever is also a human disease, usually causing a dengue-like illness, but also associated with ocular disease, encephalitis, and a hemorrhagic fever syndrome (1,2). The 1977-78 Egyptian epizootic provided a vivid demonstration of the magnitude of a Rift Valley fever epidemic in an unexposed population (1,2) and established Rift Valley fever as a threat to human as well as livestock populations worldwide (3,4). The etiologic agent of Rift Valley fever is Rift Valley fever virus (RVFV), a member of the phlebovirus genus of the Bunyaviridae family (5). The genome of RVFV consists of three RNA segments designated L, M, and S. The S segment RNA encodes the viral nucleocapsid protein N (28,000 daltons) and a nonstructural polypeptide NS_s (31,000 daltons) (6). Work carried out by us in collaboration with workers in the Virology Division, USAMRIID resulted in the molecular cloning and complete nucleotide sequence determination of the M RNA segment of RVFV, and revealed that this RNA encoded the two viral glycoproteins (G1 and G2) and possibly one or more additional, yet unidentified, nonstructural polypeptides (7). Although no direct evidence is available, it is presumed that the L segment RNA codes for the L protein, a component of the viral transcriptase.

An important goal of our studies on RVFV centers around devising a strategy and means for safe and effective disease prevention and protection measures. Under previous USAMRDC funding (DAMD 17-82-C-2008), we initiated investigations aimed at exploring the feasibility of developing a subunit immunogen, derived from the appropriate viral antigen(s) for use in vaccination against Rift Valley fever. This work involved the molecular cloning and complete nucleotide sequence determination of the M segment RNA of the ZH501 isolate of RVFV (7). Amino-terminal amino acid sequencing data derived from the two viral glycoproteins, combined with the amino acid sequence deduced from the major open reading frame found in the nucleotide sequence, allowed for the localization of the protein coding sequences for the G1 and G2 polypeptides (7). Regions encompassing these two genes were used in the construction of bacterial plasmid expression vectors that, when introduced into E. coli cells, directed the synthesis of RVFV-specific polypeptides; specifically RVFV glycoprotein G1 and G2 sequences. The resultant polypeptides were isolated from E. coli cells and evaluated for their antigenicity and immunogenicity. The microbially-produced viral glycoprotein analogues were antigenic in that they were specifically recognized by antibodies to the authentic viral proteins. Furthermore, these proteins were able to elicit the production of antibodies in laboratory

animals capable of reacting with the authentic glycoproteins. From limited animal studies, we found that mice immmunized with bacterially-produced G2 sequence-containing, but not G1 sequence-containing, polypeptide immunogens developed marginal titers of virus-neutralizing antibodies. Finally, a significant proportion of mice receiving the G2 analogue immunogens were protected from a lethal challenge of virulent RVFV. Although these results showed signs of promise, more extensive immunologic evaluation will be required to establish the full potential of these subunit immunogens.

The mission of the present research contract is to continue and extend the above described investigations, as well as to undertake new and additional basic studies relating to the molecular biology of RVFV. These are to include the further characterization of the M segment RNA, its messenger RNA, and the encoded polypeptides; the physical and antigenic characterization of glycoproteins G1 and G2; the genetic and antigenic relatedness and stability of RVFV isolates; the feasibility of producing recombinant virus vaccines; investigations into the host immune response to RVFV; and finally, exploration into features of virus-cell surface interactions. This first annual report details our progress relating to several of these objectives.

B. Characterization of the Messenger RNA (mRNA) of the RVFV M Segment. Members of Bunyaviridae appear to employ two fundamentally different strategies for gene expression: "negative sense" and "ambisense". The gene products of the S segment RNA (N and NS_S) of bunyaviruses and uukuviruses are translated from subgenomic viral-complementary RNA (8-13; negative sense). The N protein of phleboviruses is also encoded by subgenomic viral-complementary mRNA, but the NS_S polypeptide is translated from a different subgenomic viral-sense mRNA (6,14; ambisense). Expression of the M segment RNA of bunyaviruses, uukuviruses, and phleboviruses appears to proceed by the negative sense strategy (7,15,16). However, to more fully understand the replication and expression strategy of the M RNA of RVFV, we undertook studies to map the ends of the M segment mRNA. This was carried out using S1 nuclease mapping and oligonucleotide primer extension procedures. The results from these experiments revealed that the 3' end of the mRNA lacked approximately 112 nucleotides of the M genomic RNA sequences, and that the 5' end of the mRNA possessed all of the sequences present at the 3' end of the M RNA, but was further extended beyond the end of the genome by some 12-14 nucleotides of presumably cellular origin (Figure 1). These results have been published (17), and are analogous to data reported for the 3' end of the M segment mRNA of snowshoe hare (SSH) (18), and mRNA of the S segment of LaCrosse (LAC; 19), SSH (8), Uukuniemi (13), Akabane (11), and Germiston (12) viruses, and data on the 5' end of the S segment mRNA of SSH (20) and LAC (19), and the M segment of mRNA of SSH (18). Thus, it may be a general feature that mRNAs generated from

the negative sense genomic RNA are truncated at their 3' termini and may employ a "cap-scavenging" mechanism of mRNA genesis.

C. Mapping Antigenic Determinants on the G2 glycoprotein of RVFV. Definition and characterization of amino acid sequences present in select viral polypeptides that are critical for virus infectivity would be invaluable for establishing functional domains on viral proteins, for exploring certain features of viral pathogenesis, as well as for novel vaccine immunogen design. Toward this end, we have developed procedures for the localization of antigenic determinants, as defined by monolconal antibodies (mAbs), along the G2 glycoprotein (21). For this development, we initially chose four mAbs (generated and provided by the Virology Division, USAMRIID) to the RVFV G2 glycoprotein, three of which were capable of neutralizing virus infectivity (mAb I, II and IV), and the fourth (mAb III), non-neutralizing. We initially characterized the epitopes recognized by these four mAbs as either having linear or higher order structure by immunoprecipitation of denatured or native authentic antigen. We found that mAb I, II, and III were able to recognize denatured G2 glycoprotein, while mAb IV was unable to react with the denatured protein. Next, we constructed a bacterial plasmid that possessed the amino-terminal 89% of the mature G2 protein gene fused to the β-galactosidase gene in such a fashion that, when introduced into *E. coli*, this plasmid directed the synthesis of a G2-β-galactosidase fusion protein. All four mAbs reacted with this bacterially expressed G2 analogue in the same manner as they reacted with the authentic G2 glycoprotein. For epitope localization, we employed this bacterial expression plasmid/β-galactosidase fusion protein system. A nuclease BAL31 plasmid expression library was generated in which processive regions of the 3' end of the G2 glycoprotein coding sequences were deleted. Screening members of this library for mAb reactivity allowed for the approximation of the carboxy-terminal limit of the antigenic determinants. Further subcloning of limited G2 polypeptide sequences into the bacterial expression vector permitted more refined localization of the epitopes. The characteristics of the immunoreactivity of these small peptide regions (between 11 and 34 amino acids; Figure 2) produced in bacteria as G2-β-galactosidase fusion proteins were similar to those of the authentic RVFV G2 glycoprotein (21). We have subsequently localized the reactive sequences of three additional, independently derived, virus neutralizing mAbs to the same domain defined by mAbs I and IV (Figure 2). These results serve to define two distinct regions on the G2 proteins (I/IV and II) that are functionally important for virus infectivity.

D. Strategies for Protection against RVFV Infection. Two approaches toward developing a vaccine for RVF ultimately

suitable for worldwide use are currently being pursued: bacterial production of a protective subunit immunogen and use of live recombinant vaccinia viruses.

1. Bacterially-produced RVFV Glycoprotein Subunit Immunogens. Our work in this area has concentrated on producing *E. coli*-derived RVFV glycoprotein G2 analogues. Our reasons for limiting our work to G2 protein sequences are several. Mice actively immunized with authentic purified G1 protein were only poorly protected against RVFV challenge, whereas mice receiving the authentic G2 protein were clearly protected. Furthermore, only sera obtained from mice immunized with the G2 glycoprotein were able to protect naive mice upon passive immunization (J.M. Dalrymple and J. Smith, personal communications). Finally, numerous mAbs to both of the glycoproteins have been generated. Many of these mAbs, whether reactive with the G1 or the G2 protein, are capable of neutralizing virus infectivity in an *in vitro* PRNT. However, only neutralizing G2-specific mAbs are able to protect mice against RVFV challenge after passive administration (J. Smith, personal communication). In previous work we had constructed several bacterial expression plasmids that, when introduced into *E. coli*, directed the synthesis of RVFV polypeptide sequences. The polypeptides produced by two of these constructs, both possessing glycoprotein G2 sequences, are schematically shown in Figure 3B and 3C. The protein C12T1 is a 56 kilodalton polypeptide composed of 23 amino acids of the bacteriophage lambda *cro* protein, 60 amino acids of RVFV sequence immediately proceeding the G2 coding region, followed by 430 amino acids (89%) of the G2 glycoprotein (Figure 3B). Protein BH-4D (37 kilodaltons) is similar to C12T1, but possesses only the amino terminal 250 amino acids (52%) of the mature G2 protein sequence (Figure 3C). Both C12T1 and BH-4D possess all of the above described epitopes recognized by the mAbs capable of virus neutralization (Figure 2). When used as immunogens, both elicited only marginal virus neutralizing antibody titers in mice as measured by PRNT. However, a significant proportion (50-70%) of such immunized mice were protected against lethal RVFV challenge. In attempts to increase the protective immunogenicity of such *E. coli*-produced glycoprotein analogues we are exploring two avenues. One involves the evaluation of various means of polypeptide purification and preparation, and immunogen formulation. The other involves the construction of new, novel G2 protein analogues for immunologic evaluation. Our progress with this latter approach will be discussed. The genetic engineering of two new constructs has been completed. The protein JH154-8 (53 kilodaltons) possesses 207 amino acids of the mature G2 protein encompassing all of the mapped "neutralization epitopes"

and is followed by the nearly complete constant region of the heavy chain of a mouse immunoglobulin (Figure 3D). The theoretical consideration for this "Fc" construction proposes that targeting of antigens to hemopoietic cells (macrophages) possessing Fc receptors might enhance antigen immunogenicity. The second new construct is a "multicopy neutralizing epitope" polypeptide designated Nt/0/8 (Figure 3E). The sequences defined by the neutralizing mAbs have been combined into a 53 amino acid segment. In Nt/0/8, following 23 amino acids of the cro protein are eight tandemly arranged copies of this segment. The idea here is to increase the immunogenic mass of only critical peptide sequences, while eliminating sequences that may have a negative or nonessential influence on the development of protective immunity. The immunologic evaluation of these proteins is underway. However at this time, insufficient information has been collected to make any assessments of their value.

2. Live Recombinant RVFV-vaccinia Viruses. Our second approach to RVFV vaccine development has involved the construction, characterization, and evaluation of live RVFV-vaccinia viruses. We have made several such recombinants by standard procedures (22), and have characterized their ability to express RVFV protein sequences in recombinant virus-infected cells in culture. A diagram of the construction of recombinant virus vRV-5 is shown in Figure 4. Restriction with the enzyme MspI of a plasmid containing the cDNA of the complete M segment released a 3695 base pair fragment containing the entire coding regions for both RVFV glycoproteins. This fragment was inserted, with the aid of oligonucleotide linkers, into the plasmid pGS20 (22). The resultant plasmid (pvRV-5) was used to transfect cells previously infected with either the WR or Wyatt (New York Board of Health) strains of vaccinia virus. The recombinant viruses, designated vRV-5 (WR or Wyatt), were selected and purified. Cells infected with vRV-5 synthesized and correctly processed both the G2 and G1 glycoproteins of RVFV (23). Mice immunized with this live recombinant vaccinia virus developed high neutralizing antibody titers (40-2560) and were nearly completely (98%) protected upon RVFV challenge (23).

E. Research Directions for the Upcoming Year. This first annual report represents a summary of our progress in ongoing studies. We feel that our progress has been considerable and has provided several sound and potentially very rewarding avenues for continued and future investigations. Several areas that are obvious extensions of the work described in this report are briefly addressed below. Additional lines of study, as outlined in our original proposal, will be initiated as ongoing projects provide conclusions.

Now that we know the approximate structure of the M segment mRNA, we plan to carry out the cell free production of RNA molecules that as closely as possible resemble the M segment mRNA for use in *in vitro* translation studies. This work should lead to a greater understanding of the coding capacity, gene products, and expression strategy of the M segment of RVFV.

Further studies of the domains on the G2 glycoprotein defined by virus neutralizing mAbs I, II and IV will be carried out. The work will focus on the mechanism by which these mAbs neutralize virus infectivity and will explore such possibilities as their induction of virus aggregation and prevention of virus-cell interactions.

We will continue our assessment of the ability of *E. coli*-produced G2 analogue polypeptides to elicit protective immunity in laboratory mice. With the four proteins described in this report, we will be concerned with methods of protein purification, means of immunogen preparation, and mode of animal presentation.

The recombinant vaccinia virus vRV-5, or derivatives thereof, holds excellent promise for future vaccine development. The vaccinia system also has value as a model to study M segment RNA expression in mammalian cells in the absence of the L and S segment RNAs and their gene products. We plan to use this system of study for this purpose and plan to generate additional recombinant viruses to address specific experimental questions.

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FIGURE 1

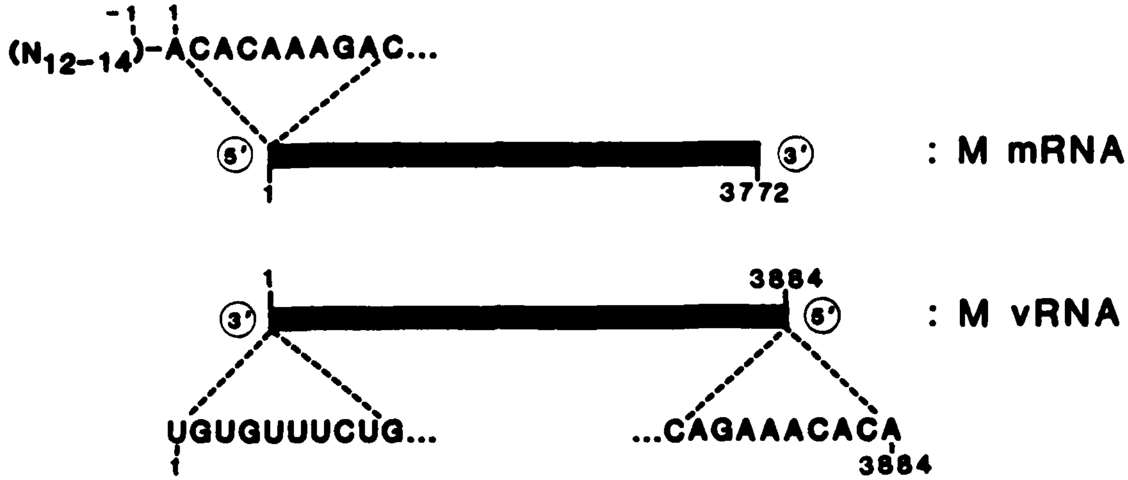
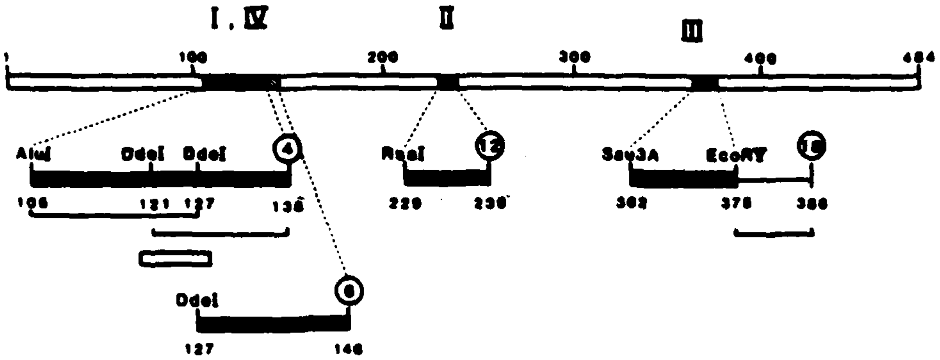


Figure 1. Diagramatic representation of the RVFV M segment genomic RNA (vRNA) and messenger RNA (mRNA). 3' and 5' denote the polarity of the RNA. The numbers represent nucleotide positions in the sequence. The terminal complementary nucleotide sequences have been expanded.

FIGURE 2

A.



B.

Epitope	Sequence
I	...HYLNNDGKMASVKCPPKYGLTEDCNFCRQMTGAS (105, 121, 127, 138)
II	...KGTMDSGQTKR (229, 239)
III	...SQSPSTEITLKYPG (362, 375)
IV	...DCNFCRQMTGASLKKGSYPL (127, 146)

Figure 2. Antigenic determinants along the G2 glycoprotein of RVFV. (A) Schematic diagram of the location of epitopes along the G2 glycoprotein. The alignment of the antigenic determinants defined by mAbs I, II, III, and IV with the mature G2 polypeptide is presented. The circled numbers represent nuclease BAL 31 expression clone numbers; all other numbers are amino acid coordinates of the mature G2 glycoprotein. Positions of restriction sites relevant to the subcloning of RVFV sequences are shown. The solid bars represent the subcloned sequences which were immunoreactive with the indicated mAb. The thin lines represent subcloned G2 polypeptide sequences that were not immunoreactive with the mAb. The open bar signifies a proposed region of immunoreactivity. (B) Amino acid sequence in the region of the mapped epitopes.

FIGURE 3

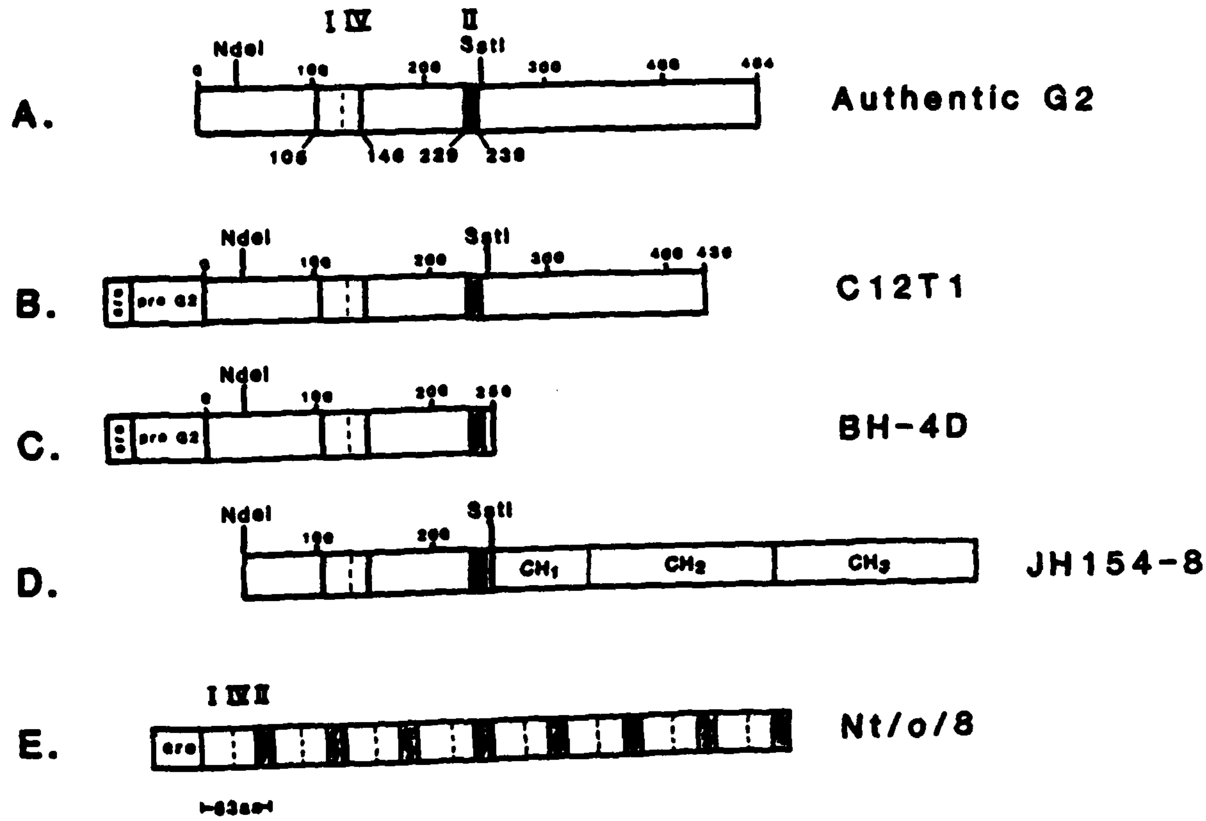


Figure 3. Schematic representations of various RVFV G2 sequence-containing polypeptides. (A) Authentic RVFV G2 glycoprotein. The numbers represent amino acid positions in the mature G2 protein. Also denoted are the bounds of the epitopes (I, IV, and II) defined by neutralizing mAbs (see Figure 2). NdeI and SstI indicate the positions of these two restriction endonuclease sites within the G2 glycoprotein gene and serve as landmarks along the sequence. (B) *E. coli*-produced recombinant protein C12T1 possesses 23 amino acids of the bacteriophage lambda *cro* protein, 60 amino acids of RVFV sequence immediately proceeding the G2 coding region, followed by 430 amino acids of the G2 protein. (C) *E. coli*-produced recombinant protein BH-4D is similar to C12T1 but possesses only the amino terminal 250 amino acids of the G2 protein. (D) *E. coli*-produced recombinant protein JH154-8 contains 207 amino acids of the G2 protein between the NdeI and SstI sites fused to the constant region of the heavy chain of a mouse immunoglobulin. C_{H1}, C_{H2}, and C_{H3} denote the various domains in the heavy chain constant region. (E) *E. coli*-produced recombinant protein Nt/0/8 has attached to the 23 amino acids of the *cro* protein, eight tandemly arranged copies of a 53 amino acid (aa) segment. This segment contains of amino acid sequences of epitopes I, IV and II (Figure 2).

FIGURE 4

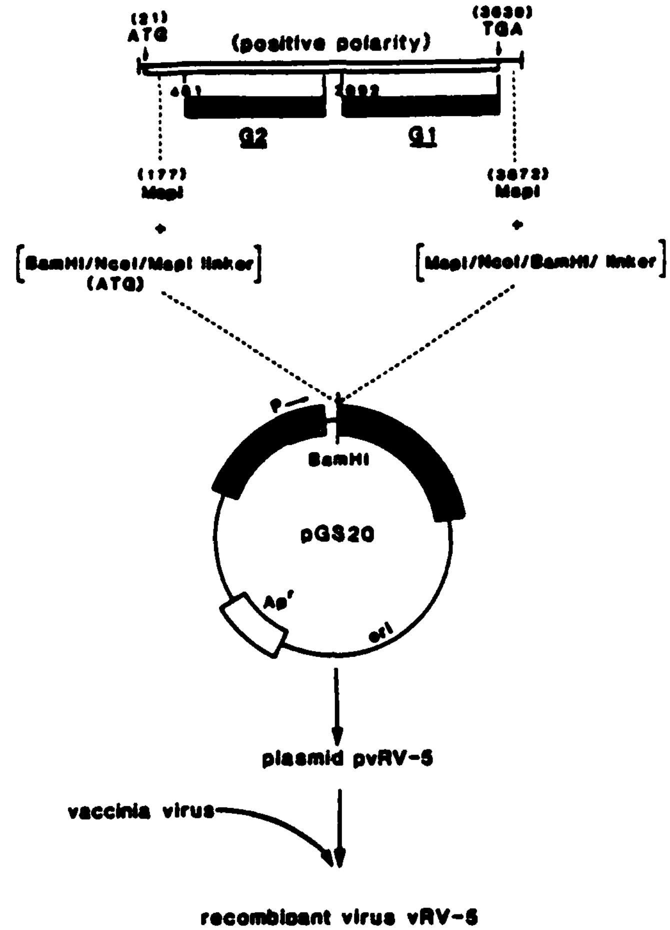


Figure 4. Recombinant RVFV M segment-vaccinia virus. A plasmid containing the entire M segment of RVFV was restricted with MspI. The released 3695 base pair fragment was isolated and ligated to BamHI-cut plasmid pGS20 (22) in the presence of two oligonucleotides. These oligonucleotides had the following sequences: 5'-GATCCACCATGGTAC-3' and 3'-GTGGTACCATGGC-5'. They are complementary over eleven continuous bases, and when combined, form a BamHI overhang at one end and an MspI overhang at the other. An optimized initiation codon (ACCATGG) is present in the middle of the first oligonucleotide, and if correctly ligated to the MspI site at position 177 in the RVFV sequence, would be in phase with the major open reading frame of the M segment. A plasmid (pvRV-5) was obtained which contained the linker oligonucleotides at either end of the RVFV MspI restriction fragment in the proper orientation with respect to the 7.5 gene promoter (P) present in pGS20. This plasmid was transfected into vaccinia virus-infected cells, and recombinant viruses were selected by previously described procedures (22). The hatched area in pGS20 represents the 7.5 gene promoter, the solid areas represent portions of the vaccinia virus TK gene, and the open box indicates the β-lactamase (Ap^r) gene.

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